STUDIES ON RIBONUCLEOPROTEIN ANTIGENS MODIFIED WITH REACTIVE OXYGEN SPECIES

DISSERTATION

SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS
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BY

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To My

Parents

CERTIFICATE

I certify that the work presented in the following pages has been carried out by **Mr. Ghulam Waris** and is suitable for the award of **M.Phil**. degree in Biochemistry of the Aligarh Muslim University, Aligarh.

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C O N T E N T S

ABSTRACT

Antibodies to saline extractable nuclear antigens (ENAs) are characteristic of certain autoimmune diseases. The most abundant of ENAs are the ribonucleoprotein (RNP) particles. These particles reside on small uridine rich RNA called URNA. Sm is present on U1, U2, U4, U5 and U6 snRNPs whereas RNP activity is mainly associated with U1snRNP. Antibodies to Sm are found almost exclusively in SLE whereas antibodies to RNP are associated with SLE and mixed connective tissue diseases (MCTD). In MCTD, the concentration of anti-RNP antibodies are considerably high and constitute one of the major criteria for the diagnosis of disease.

In the present study RNP particles were isolated from fresh goat liver nuclei by passing the nuclear extract through Sepharose 4B and DEAE Sephacel respectively. Gradient polyacrylamide gel electrophoresis of RNP particles under denaturing conditions showed polypeptides of molecular weight 29kd, 32kd, 35kd, 40kd, 45kd, 55kd, 65kd, 99kd and 116kd. The RNP particles in presence of hydrogen peroxide were exposed to UV light. The hydroxyl radical generated by the combined action of ultraviolet light and hydrogen peroxide modified the native structure of RNP particles as detected by altered pattern of UV spectra and absence of some high molecular weight polypeptides.

The structural modification was also detected by increased melting temperature of ROS-RNP ($94^{o}C$) as compared to native RNP particles ($86^{o}C$).

Binding of native RNP and ROS-RNP particles with anti-DNA positive SLE sera was studied by direct binding and competition ELISA. Direct binding studies indicated autoantibody preference for ROS-RNP particles. But when native RNP and ROS-RNP particles were tested as inhibitors it was found that ROS-RNP particles are probably not the preferred antigen for SLE autoantibody binding.

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

APS	Ammonium persulphate
BSA	Bovine serum albumin
DEAE	Diethylaminoethane
nDNA	Native deoxyribonucleic acid
EGTA	Ethylene glycol bis (β- aminoethyl ether) N,N,N',N'- tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ENA	Extractable nuclear antigen
HM	Homogenizing medium
IgG	Immunoglobulin G
MCTD	Mixed connective tissue disease
PAGE	Polyacrylamide gel electrophoresis
PMSF	Phenylmethylsulphonylfluoride
PVS	Polyvinylsulphate
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SLE	Systemic lupus erythematosus
snRNP	small nuclear ribonucleoprotein
TEMED	N,N,N',N'- tetramethylethylenediamine
Tm	Melting temperature
Tris	Tris (hydroxymethyl) aminomethane
UV	Ultraviolet

Introduction

The immune system is endowed with potential to discriminate between self and nonself and combat with the agents which are foreign or nonself (Bicker, 1986). During immunological disbalance as a result of internal threat, the body's own tissue components become reactive and may result in the initiation of autoimmune process/disease (Deodhar, 1992). The earlier contention on spontaneous origin of autoantibodies to many nuclear and cytoplasmic antigens in certain human diseases has been widely accepted now (Tan, 1989; Pisetsky, 1994). Those diseases which are characterized by the appearance of naturally occurring autoantibodies are called autoimmune diseases and include systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), Sjogren's syndrome (SS), Progressive systemic sclerosis (PSS) and mixed connective tissue disease (MCTD). The human SLE is characterized by the presence of serum autoantibodies that bind to numerous self antigens resulting in immune complex formation which subsequently damage endothelial cells and cause injury to joints and kidneys. The anti-DNA antibodies derived from SLE exhibit multiple antigenic specificities (Ali et al., 1992; Arif et al., 1994) which include components of DNA and its different conformations including modified structures (Hasan et al., 1991; Alam and Ali, 1992; Alam et al., 1995).

Aetiology of Autoimmune Diseases

Many theories have been postulated to explain the immune response(s) against self components. They include genetic factors, exaggerated random B-cell activity, cross reactivity between host and foreign antigens or modification of host antigens due to infection, drug administration, inflammation etc. (Alam et al., 1993; Ara and Ali, 1993). However the two widely accepted opinions regarding immune response against self antigens continue to gain support from research in the field of general immunology. The opinions state

(i) that an age dependent breakdown of self tolerance occurs i.e. there is less suppression of self reactive lymphocytes.

(ii) that the self reactive antibodies have a true biological function, acting as transporting agents for cellular breakdown products.

(iii) an age dependent increase in autoantibodies.

Ribonucleoprotein (RNP) Particles

Eukaryotic cells contain a group of metabolically stable uridine rich RNAs known as UsnRNAs (Reddy and Busch, 1983; Steitz et al., 1983; Brunel et al., 1985). In mammalian cells, atleast thirteen distinct snRNAs (U1-U13) have been identified (Reddy and Montzka et al., 1988). The components U3, U8 and U13

are localized in the nucleolus where they probably participate in pre-rRNA processing events (Kass et al., 1990), while the other snRNAs reside in the nucleoplasm. The major nucleoplasmic species are U1, U2, U4, U5 and U6 as shown by indirect immunofluorescence using specific antibodies directed against these RNPs (Lerner et al., 1980). These snRNAs range in size from 95 (U6) to 196(U2) nucleotides, while U1snRNA consists of 171 nucleotides. The snRNAs are associated with a set of proteins to form ribonucleoprotein particles (snRNPs) (Lerner et al., 1979). The snRNPs have been the focus of scientific interest mainly for two reasons. Namely their connection to certain autoimmune diseases and the important role which snRNPs play in the processing of nuclear pre-mRNAs (Luhrmann et al., 1990). These snRNPs are saline extractable nuclear antigens (ENA), the most abundant ENA being the Sm and RNP antigens. Sm is present on U1, U2, U4, U5 and U6 snRNPs whereas RNP activity is associated with U1snRNP.

Antibodies to saline extractable nuclear antigens are of considerable interest because their level may go to 20% of the total serum immunoglobulins in some disease(s) (Maddison and Reichlin, 1977). Such antibodies are found with high frequency in patients with rheumatic diseases. So far anti-Sm antibodies have been detected in SLE (Conner et al., 1982; Fisher et al., 1983), anti-Scl 70 in diffuse scleroderma (Jarzabek-Chorzelska

et al., 1986), anti U1RNP in SLE and MCTD (Conner et al., 1982; Fisher et al., 1983), anti Ro/SSA and anti La/SSB in SLE and in Sjogren's syndrome respectively (Lerner et al., 1981; Reddy et al., 1983; Harmon et al., 1984; Yamagata et al., 1984; Chan et al., 1986).

The Sm and RNP are the two most common ribonucleoprotein antigens (Tan and Kunkel 1966; Mattioli and Reichlin, 1971; Sharp et al., 1972) which differ in their physico-chemical characteristics such as temperature, pH lability and their differential sensitivity to RNase. RNP is sensitive and Sm is resistant to RNase (Mattioli and Reichlin, 1971; Ishaq and Ali, 1983a). The Sm and RNP antigens have also been differentiated and characterized on the basis of the type and number of their polypeptides (Hinterberger et al., 1983; Ishaq and Ali, 1983b; Steitz et al., 1983). Antibodies to Sm are found almost exclusively in SLE whereas antibodies to RNP are associated with SLE and MCTD (Tan, 1989; van Venrooij et al., 1989; Luis et al., 1992). In MCTD, the concentration of anti-RNP antibodies are considerably high and constitute one of the major criteria for the diagnosis of MCTD.

The Sm and RNP antigens can be distinguished by anti-Sm and anti-RNP sera. Douvas et al.,(1979) have reported two polypeptides of RNP with molecular weight of 13kd and 30kd. Lerner and Steitz (1979) reported seven polypeptides of molecular

weight 12kd to 35kd for both Sm and RNP, while White and Hoch (1981) have recognized a 13kd protein as Sm whereas 70 and 40kd polypeptides are associated with RNP activity. Philip et al., (1982) reported that 70kd and 13kd proteins are conserved across species line and suggested that the 40kd and 30kd bands are infact degradation products of the 70kd proteins. The 70kd RNP antigens contain two mixed charged amino acid clusters of approximately 80 and 30 amino acid long which are particularly rich in serine, arginine, lysine, glutamic acid and aspartic acid residues. Recently it has been reported that the basic amino acids predominate in the sequential autoantigenic determinants of the 70kd RNP molecule (James et al., 1994). Sm snRNP is composed of a single snRNA and a number of unique and common proteins (van Venrooij et al., 1989; Luhrmann, 1990). Sm snRNP polypeptides can be divided into three classes according to their amino acid sequence and length.

(i) One type is Sm-B′/B which is 240 amino acids long, and have a predicted molecular weight of 24.6 kd. The deduced amino acid sequence is rich in proline (20%) and glycine (15%) (Schmauss et al., 1989).

(ii) Another class is constituted by the Sm-B′ polypeptide which is 240 amino acids long and have a 93% identity with the Sm-B′/B (van Dam et al., 1989).

(iii) The third class is constituted by the Sm-B polypeptide which is 231 residues long with molecular weight of 23.7 kd (van Dam et al., 1989).

There are two major Ro proteins of 60kd and 52kd which are acidic in nature. The human 60kd Ro protein is associated with four distinct uridine rich RNAs ranging in size from 80 to 112 nucleotides (Wolin et al., 1984). Moreover, the human 52kd Ro molecule is antigenically and structurally different from the 60kd molecule (Ben-Chetrit et al., 1988). La antigen is a 50kd phosphoprotein found principally in cell nucleus. It is associated with cellular RNAs. Binding of RNA to La depends on presence of uridylate residues at the 3' end (Mathews and Francoeur, 1984; Stephano et al., 1984) . The function of La is to facilitate the termination of transcription of RNA polymerase III (Gottlieb et al., 1989).

Autoantibodies to soluble cellular protein antigens, Ro ribonucleoprotein particle (Ro/SSA) have been found primarily in Sjogren's syndrome (SS), systemic lupus erythematosus (SLE) and in subset of lupus patients (Harley et al., 1986; Tan, 1989). Anti-Ro antibodies have been found in various percentages of sera from patients with SLE (12-69%), SS (38-96%), rheumatoid arthritis (15-28%) (Tan, 1989; Slobbe et al., 1991). Autoantibodies against SS-B ribonucleoprotein (SS-B/La) antigen have been detected in sera of patients with primary Sjogren's syndrome, systemic lupus erythematosus, rheumatoid arthritis and undifferentiated connective tissue diseases (Akizuki et al., 1977; Pfeifle et al., 1986). Ro/SSA is found within cytoplasm

whereas La/SSB has been thought to reside in the nucleus but leaks out during preparation of cell fractions (Clark et al , 1968, Akizuki et al., 1977) Ro/SSA antibodies have been closely associated with the appearance of nephritis, vasculitis, lymphadenopathy and leukopenia in SLE patients (Maddison and Reichlin, 1976; Alexander et al., 1983).

Biological Functions of RNP Particles

Many eukaryotic genes which encode mRNA are separated by introns Expression of these genes requires the excision of introns from the primary transcript called pre-mRNA splicing They are not self splicing but require the action of specialized RNA-protein complex called RNP complex In UsnRNPs, U1, U2 and U4/U6 snRNPs are essential splicing factors (Kramer et al , 1984; Black et al., 1985, Berget, 1986) The U1snRNA has a sequence complementary to sequence near the 5' splicing site of the nuclear mRNA introns and the U1snRNP binds to this region in the primary transcript (Lerner et al , 1980). U2snRNP binds to the branch point of the introns U4/U6 and U5snRNPs are assembled into the spliceosome and interact with pre-mRNA bound U1 and U2snRNPs rather than interacting pre-mRNA sequence directly (Bindereif et al , 1986)

Reactive Oxygen Species in Relation to Biology and Medicine

The term reactive oxygen species (ROS) is a collective one that includes not only oxygen centred radicals such as

superoxide anion ($O_2^{\cdot-}$) and hydroxyl radical ($OH^{\cdot}$), but also some non-radical derivative of oxygen, such as hydrogen peroxide (H_2O_2), singlet oxygen, hypochlorous acid (HOCl) and ozone (O_3). Hydroxyl radical is produced in living organisms by at least two mechanisms - reaction of transition metal ions with H_2O_2 and homolytic fission of water due to background exposure to ionizing radiation (von Sonntage, 1987). Hydroxyl radical is an extremely reactive species that can attack all biological molecules usually setting off free radical chain reactions (Halliwell and Gutteridge, 1989). Superoxide radical ($\dot{O}_2^-$) is much less reactive than hydroxyl radical, but a number of biological targets can be attacked by it. Superoxide anion can act as a vasoconstricter and this may have deleterious effects under some clinical conditions (Laurindo et al., 1991). Superoxide may inactivate the NADH dehydrogenase complex of the mitochondrial electron transport chain (Zhang et al., 1990). Some of the superoxide anion production that occur <u>in vivo</u> appears to be due to autooxidation reaction and leakage of electrons from electron transport chain (Fridovich, 1989; Imlay and Fridovich, 1991).

Hydrogen peroxide is a normal metabolite in aerobic cells formed by reduction of dioxygen or by dismutation of the superoxide anion radical (Tachon, 1989). In steady state, the concentrations of hydrogen peroxide is in the range of 10^{-8}M to

10^{-9}M (Oshino, 1973). Hydrogen peroxide is a relatively stable oxidant and does not cause macromolecule damage by itself. In intact cells it is the precursor of a much more damaging reactant (Repine et al., 1981). This very reactant may be the hydroxyl radical. However, its production can be increased under several pathological conditions, especially at the site of inflammation where the oxidative burst of the phagocytic cells occur (Hammers and Roos, 1985) and upon irradiation with near ultraviolet or visible light (Hoffman and Meneghine, 1979) and ionizing radiations (Hart, 1972). Hydrogen peroxidee crosses cell membranes easily and it can attack, apparently directly, a few cellular targets.

Several transition metal salts react with H_2O_2 to form hydroxyl radical. The chemical process that produces it can be expressed as the Haber-Weiss reaction (1934).

$$H_2O_2 + O_2^{\cdot -} \text{-------} OH^{\bullet} + OH^{-} + O_2$$

The above reaction catalyzed in the presence of iron is known as Fenton reaction (1893).

$$O_2^{\cdot -} + Fe^{+3} \text{-------} Fe^{+2} + O_2$$

$$Fe^{+2} + H_2O_2 \text{-------} Fe^{+3} + OH^{\cdot} + OH^{-}$$

$$O_2^{\cdot -} + H_2O_2 \text{-------} OH^{\cdot} + OH^{-} + O_2$$

Reactive oxygen species are known to damage a variety of biological macromolecules and have been implicated in the initiation of many human diseases (Halliwell and Gutteridge, 1990). In chronic inflammatory diseases, such as rheumatoid arthritis and SLE, the phagocytic cells release reactive oxygen species into extra cellular environment which may penetrate cell membrane and react with DNA (Stollar, 1981; Allan, 1988). Subsequent release of this altered DNA may act as an immunogen against which anti-DNA antibodies may be generated. ROS modified DNA has been proposed as better antigen for SLE anti-DNA antibodies (Blount et al., 1989; Blount et al., 1990). The ROS modified DNA have induced anti-DNA antibody response with properties exhibited by anti-DNA autoantibodies (Ara and Ali, 1992; Alam et al., 1993).

Antioxidants and Hydroxyl Radical

Reactive oxygen species can attack and irreversibly damage a diverse spectrum of biomolecules including proteins, nucleic acids, phospholipids and sugars. Aerobic organisms have potent antioxidant defences to minimize the cytotoxic effects of reactive oxidants. Superoxide dismutase (SOD) inactivates $O_2^{\cdot-}$, catalase, glutathione peroxidase takes care of hydrogen peroxide (Fridovich, 1978; Halliwell and Gutteridge, 1986). The cells also possess non-enzymatic antioxidant mechanisms for scavenging hydroxyl radical. Some of these scavengers are in the

hydrophilic phase (ascorbate, urate, glutathione) and others in the lipid phase (α-tocopherol, β-carotene). Lidocaine, a local anaesthetic has been found to be a potent scavenger of hydroxyl radicals and singlet oxygen (Das and Misra , 1992). It has been widely accepted that oxygen toxicity is normally held in check by a balance between the rate of formation and destruction of reactive forms of oxygen (Fridovich, 1977).

Protein Damage by Reactive Oxygen Species

The free radical damage to proteins consist broadly of modification of amino acid residue side chain cross linking and fragmentation. The radiation studies indicate that certain amino acids were more susceptible than others, these being the aromatic amino acids and cysteine. Hydroxyl radicals will attack protein molecules almost non-specifically with some preference for the aromatic amino acids and cytosine. Hydroxyl radical formation is most likely to be catalysed by interaction of H_2O_2 with a transition metal ion bound to some specific site on the protein and will damage amino acid residues at or near the metal binding site (Stadtman et al., 1991). Modification of amino acid residue includes deamination, carbonyl formation, oxidation of methionine to its oxide. Carbonyl formation has been extensively studied by Stadtman (1990). Examples of residue modifications include conversion of proline and histidine residues to glutamate and aspartate respectively (Dean et al.,

1989). One of the major types of free radical damage to protein is cleavage of peptide bonds and fragmentation of the polypeptide chains. Hydroxyl radical can fragment and cross link proteins (Schuessler et al., 1984). In the absence of molecular oxygen, hydroxyl radical induces cross links in protein which are often resistant to reduction.

The modification of amino acid residues will affect the general properties of the protein such as change in charge, hydrophobicity and conformations. These changes can lead to even aggregations of the molecule (Davies et al., 1987).

Nucleic Acid Damage by Reactive Oxygen Species

Hydroxyl radical mediated damage to nucleic acids is well documented (Rhaese and Frees, 1968; Schweitz, 1969; McDonald et al., 1993). It has been suggested that H_2O_2 mediated DNA damage occurs at the metal binding site (Czapski, 1984). Hydroxyl radical may attack DNA at either sugar or base (Hutchinson, 1985) causing strand breaks and modifications of DNA components. Free radicals have also been reported to aid in DNA-protein cross-linking between thymine and tyrosine (Olinski et al., 1992) and cytosine and tyrosine (Dizdaroglu et al., 1991). Apart from causing base damage in DNA, hydroxyl radicals also react with deoxyribose sugar moiety of the nucleic acids (Zhao and Jung, 1994). The mechanism of single strand break

involves addition of OH· across -C=C- double bond of the base or abstraction of H atom from sugar (Schulte et al., 1985). Other hydroxyl damaged product include pyrimidine cyclobutane type dimers (Sage, 1993). Numerous assays exist for quantification of free radical mediated DNA damage (Hageman et al., 1992). DNA damage of thymine dimers type has been detected by monoclonal antibodies (Mizeuno et al., 1991; Potten et al., 1993).

Objectives of the Present Study

The main objective of the present work was to study the effect of hydroxyl modification on the antigenic property of RNP particles binding to autoantibodies derived from SLE sera. The RNP antigen was mixed with hydrogen peroxide and exposed to UV light of 254 nm. The hydroxyl modified antigen was characterized by UV spectroscopy and thermal denaturation studies. The role of hydroxyl modified RNP in the pathogenesis of SLE has been discussed.

Experimental

A. Materials

1. Chemicals

Calf thymus DNA, bovine serum albumin, coomassie brilliant blue R 250, agarose, ethidium bromide, anti-human IgG alkaline phosphatase conjugate, Phenyl methyl-sulfonyl fluoride (PMSF), polyvinyl sulphate (PVS), orcinol, spermine and spermidine were purchased from Sigma Chemicals, USA. Ethylene glycol bis (B-amino ethyl ether)-N, N, N′, N′-tetraacetic acid (EGTA) was purchased from Koch-light laboratories, England. Polystyrene flat bottom ELISA plates (96 wells) were obtained from NUNC, Denmark. Acrylamide, bisacrylamide, N, N, N′, N′- tetramethyl ethylenediamine, Tween-20, ammonium persulphate were obtained from Bio-Rad, USA. Para-nitrophenyl phosphate was a product of CSIR Centre for Biochemicals, Delhi. Sepharose 4B, Blue dextran 2000, Sephadex G-200, Ficoll-400 and DEAE Sephacel were obtained from Pharmacia Fine Chemicals, Sweden. Nonidet P-40 was from BDH, England. Silver nitrate was from Glaxo, Bombay. RNA was isolated from buffalo thymus. All other chemicals used were of highest grade available.

2. Equipments

UV-Visible 240 recording spectrophotometer (Shimadzu,Japan), microplate reader (MR 600) (Dynatech USA), horizontal electro-

phoresis assembly (Pharmacia, Sweden), vertical electrophoresis apparatus (Bio-Rad, USA.), Fluorescent table and UV lamp of 254 nm wavelength (Vilber-Lourmat, France), pH meter (ELICO, India) were the major equipments used in the present study.

3. Serum samples

Normal human sera were obtained from healthy subjects. Sera of patients with systemic lupus erythematosus (SLE) and other autoimmune diseases were obtained from outdoor and indoor patients of the Department of Medicine, All India Institute of Medical Sciences, New Delhi and J.N. Medical College, A.M.U. Aligarh. All sera were stored at -20°C with 0.1 percent sodium azide as preservative until examined. Serum samples were decomplemented before use by heating at 56°C for 30 min.

4. Organ

Fresh goat liver obtained from slaughter house was immediately frozen in ice-sodium chloride mixture and transported to the laboratory.

B. Methods

1. Determination of Protein Concentration

Protein was estimated by the method of Lowry et al. (1951) as described below.

a. Folin-Ciocalteau Reagent

The reagent was purchased from CSIR Centre for Biochemicals, Delhi and diluted 1:4 with distilled water before use.

b. Alkaline Copper Reagent

The constituents of alkaline copper reagent were as follows:

i. 2% sodium carbonate in 0.1 N NaOH

ii. 0.5% copper sulphate in 1% sodium-potassium tartarate

The working reagent was prepared by mixing 50 ml of (i) with 1 ml of (ii) and used immediately.

c. Procedure

One ml of protein sample in appropriate buffer was mixed with 5.0 ml of alkaline copper reagent and kept at room temperature for 10 min. At the end of incubation one ml of Folin Ciocalteau reagent was added and the contents were further incubated for 30 min. The absorbance of developed colour was read at 660 nm. Protein content of the unknown samples were computed from the standard plot drawn using different concentration of bovine serum albumin (Fig 1).

2. Determination of DNA concentration

Colorimetric estimation of DNA was carried out by the method of Burton (1956) using diphenylamine reagent.

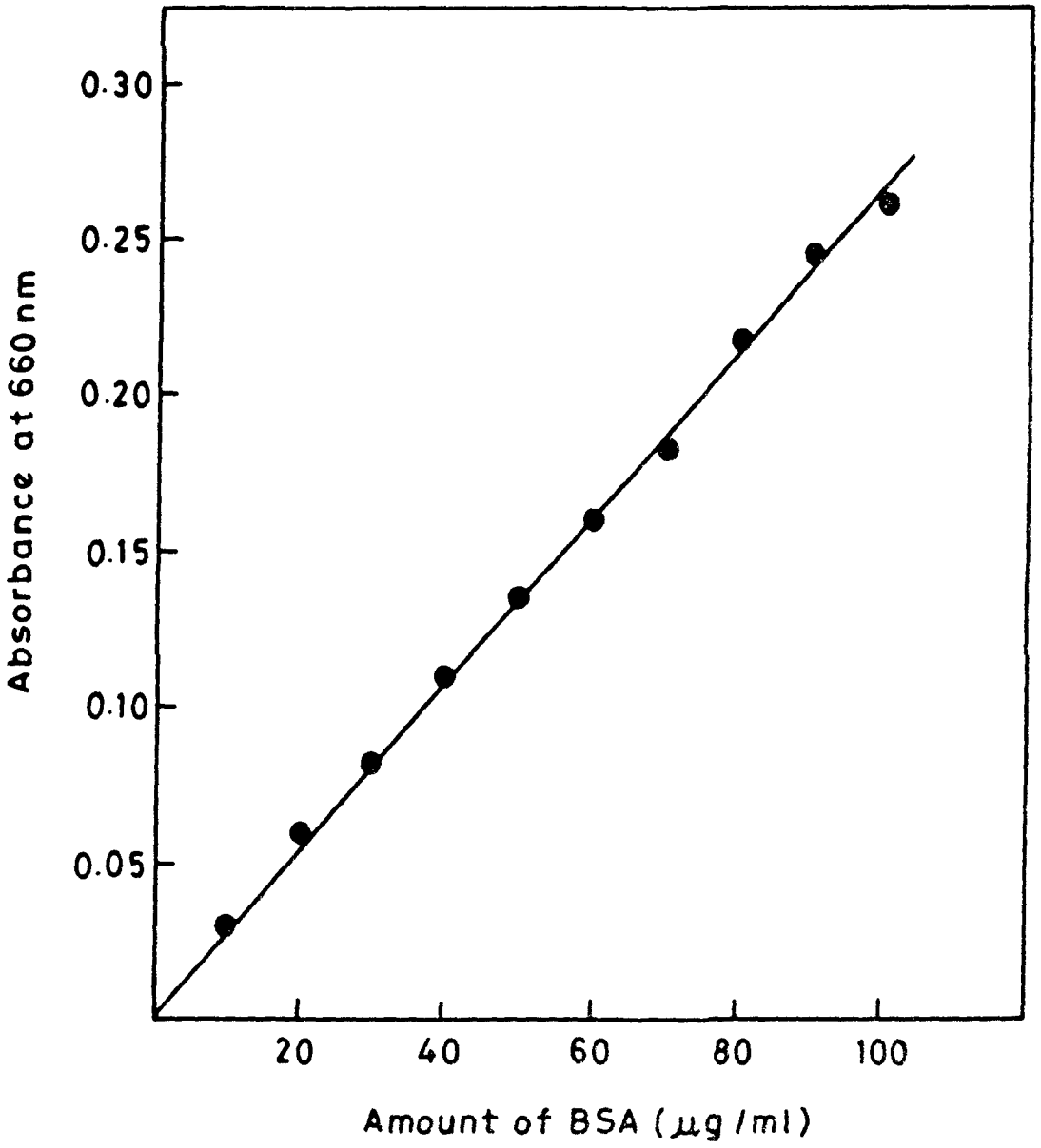


Fig. 1. Standard plot for the colorimetric estimation of protein.

a. Diphenylamine reagent

To 750 mg of diphenylamine was added 50 ml of glacial acetic acid followed by 0.75 ml of concentrated sulphuric acid. The reagent was prepared immediately before use.

b. Procedure

One milliliter DNA sample was mixed with 1 ml of 1N perchloric acid and tubes were kept at 70°C for 15 min in a thermostat water bath. Then 0.1 ml of 5.43 mM acetaldehyde was added followed by 2 ml of diphenylamine reagent. The contents were mixed and allowed to stand at room temperature for 16-20 hours. The developed colour was read at 600 nm. The DNA concentration in unknown sample was determined from standard plot of calf thymus DNA (Fig. 2).

3. Determination of RNA concentration

RNA was estimated by ferric ion catalysed orcinol reaction (Ceriotti, 1955).

a. Crystallization of Orcinol

Five gm of commercial orcinol was dissolved in 100 ml boiling benzene and decolorized with 1 gm of activated animal charcoal. The suspension was filtered while hot and kept at room temperature for 1 hour and then at 4°C until the

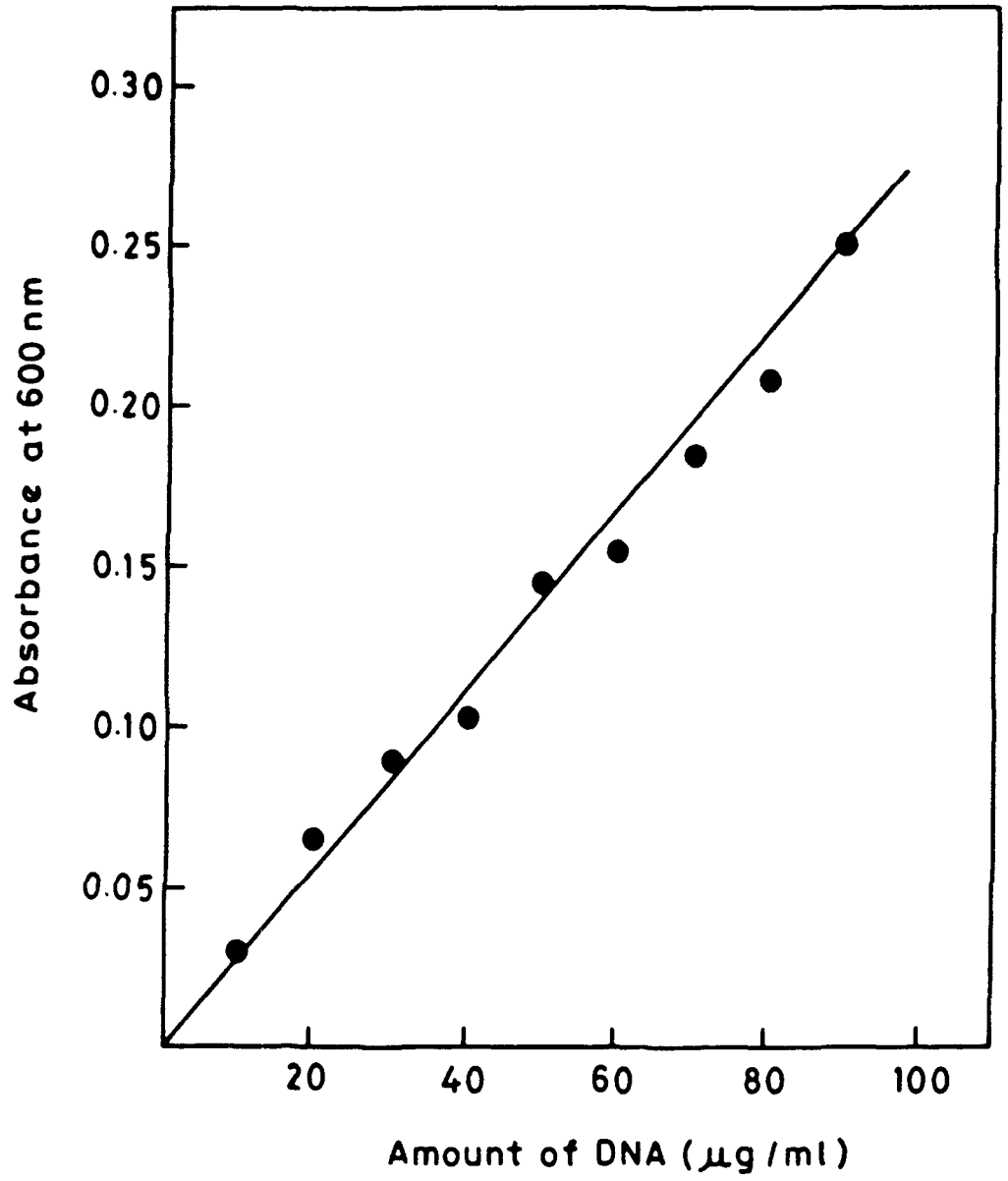


Fig. 2. Standard plot for the colorimetric estimation of DNA.

crystallization was complete. The crystals were separated by filtration and dried at room temperature.

b. Orcinol reagent

i. 100 mg ferric chloride was dissolved in 100 ml concentrated HCl

ii. 6% (w/v) orcinol in distilled ethanol

The reagent was prepared fresh by mixing 3.5 ml of (ii) with 100 ml of (i) and used immediately.

c. Procedure

Two milliliter of RNA solution was mixed with 3 ml of orcinol reagent and placed in boiling water for 30 min. The tubes were chilled in ice water and absorbance was measured at 665 nm. RNA concentration of unknown samples were calculated from the standard plot of buffalo thymus RNA (Fig.3).

4. Isolation of Immunoglobulin G (IgG)

Serum IgG was isolated by ion exchange chromatography and purified on Sephadex G-200 column.

a. Preparation of crude immunoglobulins

To 6.5 ml of decomplemented serum was added dropwise saturated ammonium sulphate solution allowing each drop to

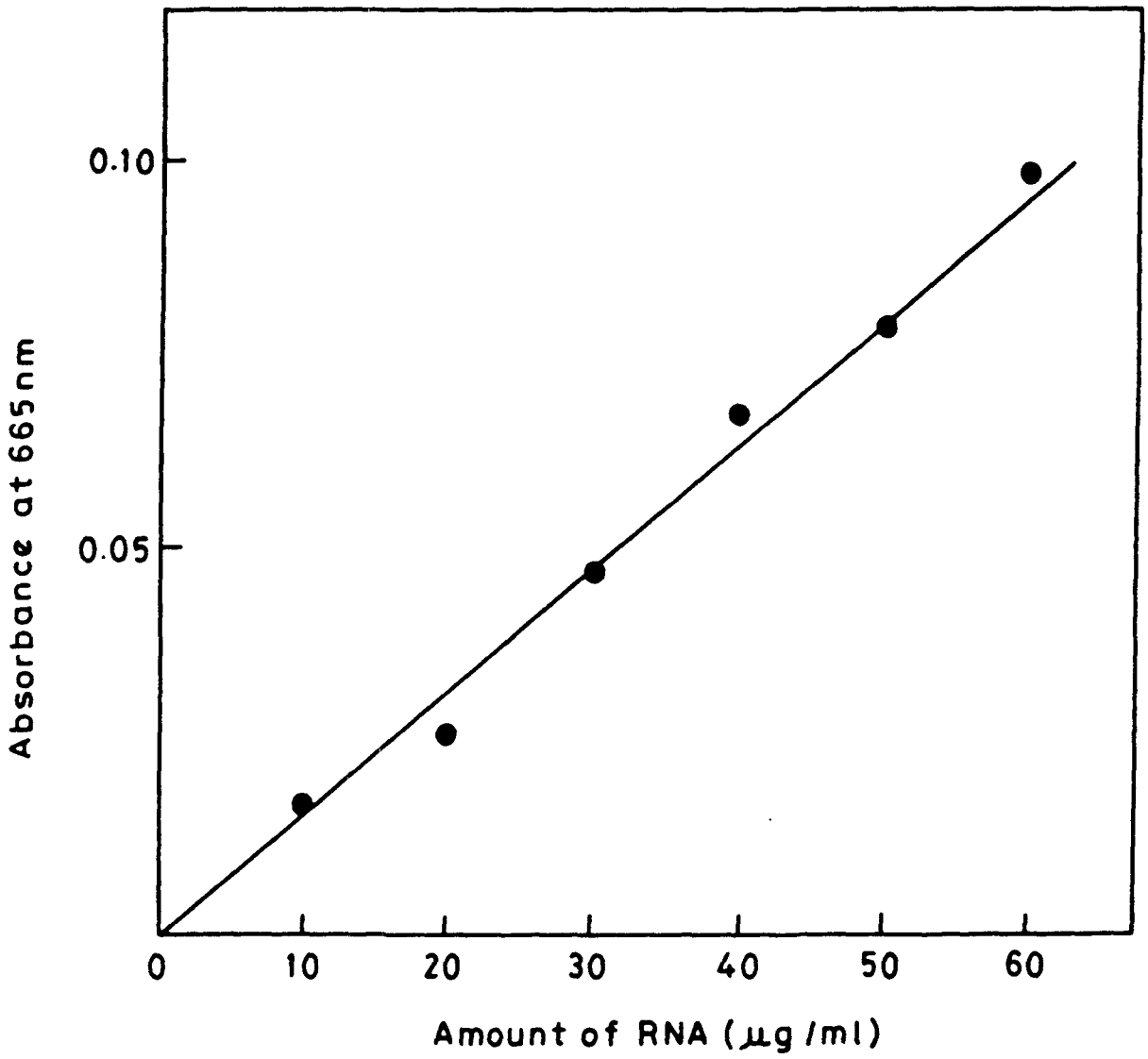


Fig. 3. Standard plot for the colorimetric estimation of RNA.

disperse before the next was added. When the concentration of ammonium sulphate reached about 20% of saturation the serum had begun to turn milky. Most immunoglobulins got precipitated by 35-37% of saturation. The reaction was carried out at 4°C. The suspension was stirred for 30 min and then centrifuged at 10,000 rpm for 15 min. The pellets thus obtained was washed two or three times in 35% saturated ammonium sulphate. Repeated washing of the pellet considerably reduced contamination with non-immunoglobulin proteins and did not lower the yield significantly. Finally the precipitate was dissolved in phosphate buffered saline (PBS, pH 8.0). Ammonium sulphate was removed by overnight dialysis against 500-1000 volumes of 0.0175 M sodium phosphate buffer, pH 6.8.

b. DEAE Sephacel ion exchange chromatography

Dialysed crude immunoglobulins were loaded on the top of a column of DEAE Sephacel (20 cm x 1.5 cm) previously equilibrated with the same buffer. The column was eluted with 0.0175 M sodium phosphate buffer, pH 6.8. Fractions of 3.0 ml were collected and monitored for protien content at 280 nm, assuming 1.4 $O.D._{280}$ = 1 mg/ml IgG.

The first peak of the chromatogram was found to contain IgG (Fig. 4). The homogeneity of isolated IgG was checked by single band movement in SDS-PAGE.

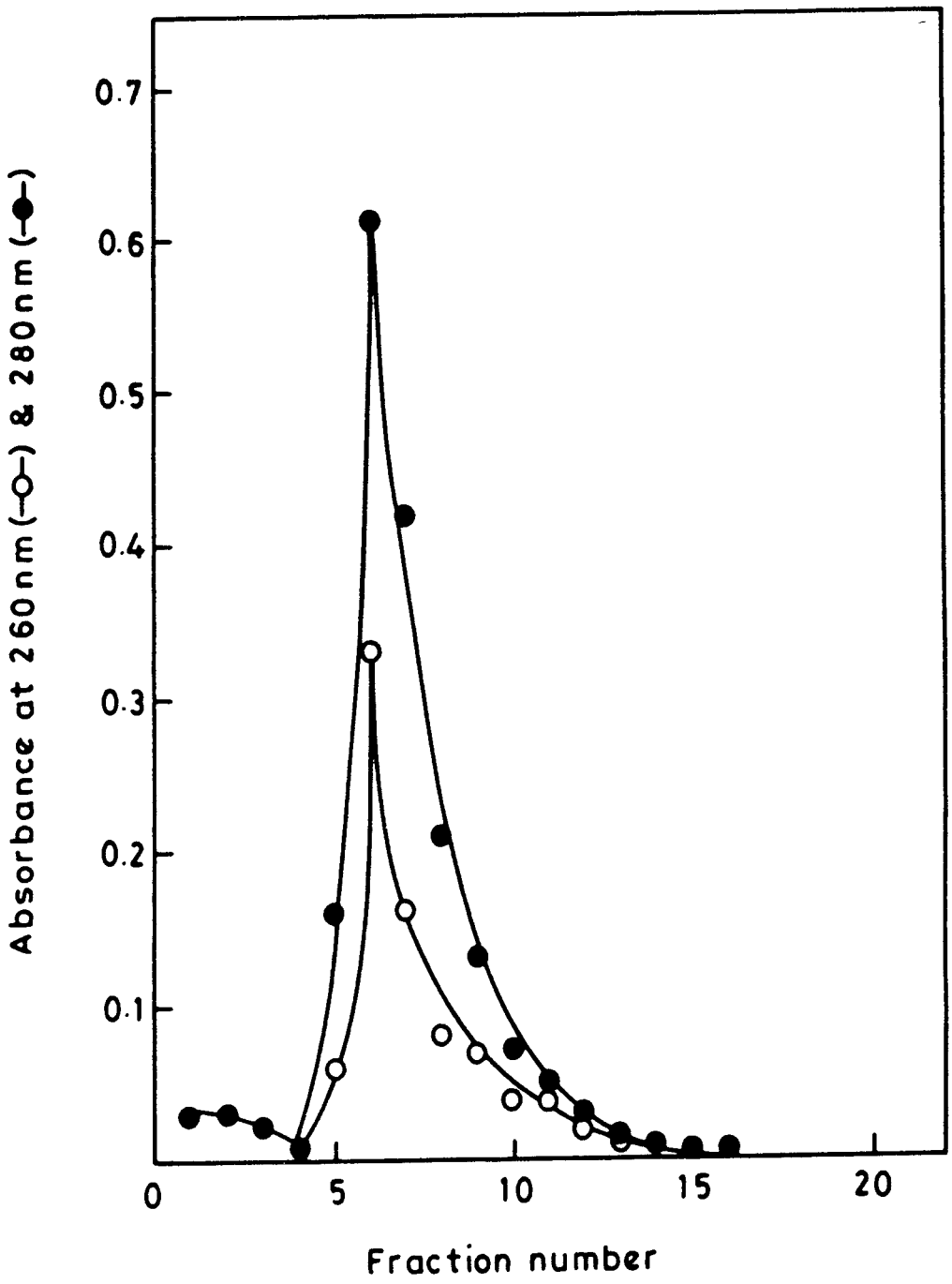


Fig. 4. Elution profile of IgG from normal human serum passed through DEAE Sephacel column.

c. Isolation of IgG by gel filteration method

Crude immunoglobulins were loaded on a gravity packed Sephadex G-200 column (75 cm x 2 cm) previously equilibrated with 0.0175 M sodium phosphate buffer, pH 6.8. The column was operated at a flow rate of 25 ml/hour and fractionated immunoglobulins were monitored for protein at 280 nm. The IgG peak was identified by absorbance measurements at 278 and 251 nm. Mammalian IgG absorbance at 278 nm is about 2.5 to 3.0 times higher than at 251 nm, in contrast to other serum proteins where this ratio is about 1-1.5 (in neutral buffers). (Fig. 5).

5. Polyacrylamide Gel Electrophoresis (PAGE)

The purity of isolated IgG was checked through polyacrylamide slab gel electrophoresis under denaturing conditions as described by Laemmli (1970).

i. Stock Solutions

a. Acrylamide - bisacrylamide (30:0.8)

The stock solution was prepared by dissolving 30 gm acrylamide and 0.8 gm bisacrylamide in a total volume of 100 ml. As far as possible , the mixture was protected from light.

b. Resolving gel buffer, 3 M Tris-HCl

Stock solution of buffer was prepared by dissolving 36.3 gm Tris in 48.0 ml of 1N HCl. The contents were mixed thoroughly, pH adjusted to 8.8 and volume brought to 100 ml.

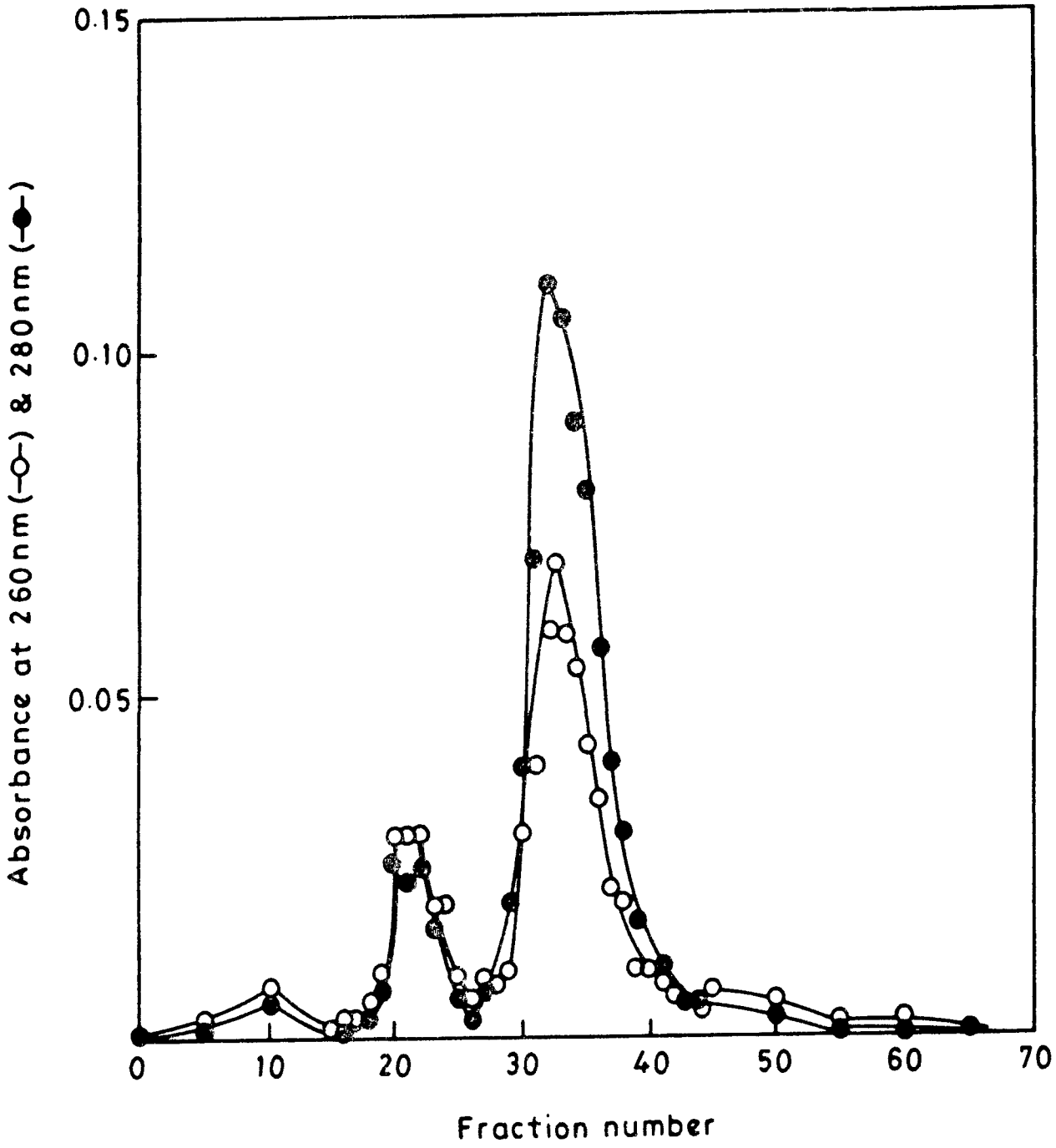


Fig. 5. Profile of normal human IgG on Sephadex G-200 column. Inset: Polyacrylamide gel electrophoresis showing the homogeneity of isolated IgG.

c. Stacking gel buffer, 0.5 M Tris-HCl

6.05 gm Tris was dissolved in 40 ml of distilled water, titrated to pH 6.8 with 1N HCl (~48 ml) and volume adjusted to 100 ml with distilled water.

ii. Electrode buffer

0.025 M Tris and 0.192 M glycine,pH 8.3 containing 0.1% SDS.

iii. Sample buffer

a. Six gm Tris was dissolved in 80 ml distilled water and pH adjusted to 6.8 with phosphoric acid . The volume was made to 100 ml with distilled water.

b. To 12.5 ml of above sample buffer, was added 1 mg bromophenol blue and 12.5 ml glycerol. One part of (b) and four part of sample were mixed and boiled for five min. prior to loading.

iv. Procedure

Glass plates separated by spacer of appropriate thickness were sealed with agarose dissolved in resolving gel buffer. The polymerizing mixture was poured between the glass plates and left at room temperature for complete polymerization. Protein samples were prepared and loaded in wells. The gel was run for 8-10 hours at 80 volts. At the end of electrophoresis the gel was stained with coomassie brilliant blue dye.

RECIPE FOR 7.5% SDS-PAGE

(Total volume 40.0 ml)

Acrylamide - bisacrylamide	-	10.0 ml
Resolving gel buffer	-	5.0 ml
10% SDS	-	0.4 ml
1.5% Ammonium persulphate	-	2.0 ml
Distilled water	-	22.6 ml
TEMED	-	0.02 ml

RECIPE FOR 5-20 % GRADIENT GEL

(Total volume 24.0 ml)

	5 % gel	20 % gel
Acrylamide-bisacrylamide	2.0 ml	8.0 ml
Resolving gel buffer	1.5 ml	1.5 ml
10 % SDS	0.1 ml	0.1 ml
1.5 % Ammonium persulphate	0.6 ml	0.6 ml
Distilled water	7.774 ml	1.774 ml
TEMED	0.006 ml	0.006 ml

6. Isolation and Purification of Ribonuleoprotein (RNP) Antigens

Ribonucleoprotein antigen was isolated from goat liver nuclei. All procedures were carried out at 0-4°C. Acid washed glassware and sterilized solutions were used to prevent nuclease and protease activity.

Homogenizing medium

Homogenizing medium (HM) contained 250 mM sucrose 5 mM magnesium acetate, 0.1 mM ethylene glycol bis (β-amino ethyl ether)-N,N,N′N′-tetraacetic acid (EGTA) and 5 mM 2-mercaptoethanol. In addition , it contained 1 mM phenyl methyl sulphonyl fluoride (PMSF) , 1 mM spermine, 1 mM spermidine and 10 µg/ml polyvinyl sulphate (PVS). PMSF was dissolved in minimal volume of isopropyl alcohol and then added to HM immediately before use. HM-NP40 contained all the above components plus 0.5 percent (v/v) Nonidet P-40.

a. Isolation of nuclei

Nuclei were isolated essentially by the method of Douvas et al (1979) with slight modifications. Briefly frozen liver was thawed in HM for 20-30 min. The thawed tissue was cut into small pieces and homogenized with chilled HM for short period to avoid nuclear breakdown. The homogenate was filtered through 3-4 layers of cheese cloth. The filtrate was

centrifuged at 2500-3000 rpm for 10 min. The pellets were washed twice and extracted with HM-NP40 for 30 min. using magnetic stirrer. The suspension was centrifuged and the pellets were washed thrice with HM-NP40. The final washing of the pellets were performed with HM.

b. Extraction of nuclei

Nuclear pellets were extracted four times with STM buffer (10 mM Tris-HCl, 100 mM NaCl, 1 mM $MgCl_2$ containing 1 mM PMSF and 10 µg/ml polyvinyl sulphate, pH 8.0). Each extraction (end to end) was carried out for 1-2 hours. The insoluble material was separated by centrifugation and supernates pooled together and dialysed against STM buffer to remove sucrose. The insoluble material formed during dialysis was centrifuged out and the supernatant designated as nuclear extract was used as the source of ribonucleoprotein (RNP) antigens.

c. Sepharose 4B chromatography

The nuclear extract was passed through a column of Sepharose 4B (70 cm x 2 cm) earlier equilibrated with STM buffer. Elution was carried out with STM buffer. Fractions of 3 ml were collected and monitored for protein, RNA and DNA contents. The fractions containing protein were pooled and dialysed against 10 mM sodium phosphate buffer pH 7.3 containing 50 mM NaCl.

d. DEAE Sephacel chromatography

The dialysed antigen was loaded onto a column of DEAE Sephacel (6 cm x 1.5 cm) washed and equilibrated with 10 mM sodium phosphate buffer, pH 7.3 containing 50 mM NaCl. The ribonucleoprotein antigens were eluted with 10 mM sodium phosphate buffer, pH 7.3 containing 300 mM NaCl.

7. Irradiation of Ribonucleoprotein (RNP) Antigens

Ribonucleoprotein antigen (0.00145 mM) in PBS (0.01 M sodium phosphate buffer containing 0.15 M NaCl, pH 7.4) were irradiated under 254 nm UV light for 30 minutes at room temperature in presence of hydrogen peroxide. The molar ratio of RNP to hydrogen peroxide was 1:100. RNP exposed to UV light in the absence of hydrogen peroxide served as control. RNP - hydrogen peroxide complex was also included as internal control.

8. Thermal Denaturation of RNP Antigens

Melting curves of unmodified and modified ribonucleoprotein antigen was recorded on spectrophotometer equipped with melting device as described earlier (Hasan and Ali, 1990). Samples were melted from 30°C to 95°C at a rate of 1.5°C per min. The wavelength was fixed at 280 nm. Percent denaturation was calculated as follows

$$\text{Percent denaturation} = \frac{(A_x - A_{30}) \text{ at various temperature}}{(A_x - A_{30}) \text{ at } 95°C} \times 100$$

Where, A_x = absorbance value at a particular temperature

A_{30} = absorbance value at 30°C.

9. SDS-PAGE of Ribonucleoprotein (RNP) Antigens

The RNP antigens were dialysed against 20 mM Tris-HCl (pH 7.4) buffer containing 2 mM EDTA, 5 mM 2- mercaptoethanol, 0.1% SDS and electrophoresed on 5-20% gradient gel. The gel mixture was poured between the plates and allowed to polymerize at room temperature. Two hundred microgram RNP antigen was loaded and run for 8-10 hrs at 80 volts at room temperature. At the end of electrophoresis the gel was stained with coomassie brilliant blue R250 (0.25% in 10% acetic acid and 25% methanol). The visible bands were photographed and recorded.

10. Agarose Gel Electrophoresis of RNP Antigens

Agarose was dissolved at a concentration of 0.4% in electrophoresis buffer (40 mM Tris-acetate, pH 8.0 containing 2 mM EDTA) and poured in tray fitted with combs. RNP antigens were loaded in the wells and electrophoresed for 2-4 hours at 30 mA. At the end of process the gel was stained in ethidium bromide and visualised for RNA under UV light.

11. Direct Binding ELISA

The following reagents were prepared in distilled water and used in enzyme immunoassay.

a. Tris buffered saline (TBS)

10 mM Tris, 150 mM NaCl, pH 7.4 containing 0.02% sodium azide as preservative.

b. Tris buffered saline - Tween 20 (TBS-T)

20 mM Tris, 144 mM NaCl, 2.68 mM KCl pH 7.4 containing 500 µl Tween-20 in one litre.

c. Bicarbonate buffer

15 mM sodium carbonate, 35 mM sodium bicarbonate and 2 mM magnesium chloride, pH 9.6 containing 0.02% sodium azide as preservative.

d. Substrate

500 µg para-nitrophenyl phosphate in 1 ml bicarbonate buffer.

Procedure

SLE autoantibody binding to DNA, RNP and ROS-RNP in SLE were detected by ELISA. Anti-DNA antibody, were detected on

microtiter wells previously coated with 100 ul of poly D-lysine (50 µg/ml in distilled water) for 30 min at room temperature. The plate was washed thrice with TBS and coated with 100 µl of DNA (2.5 µg/ml in TBS) for 2 hours at room temperature and overnight at 4°C. The antigen coated wells were washed thrice with TBS-T to remove the unbound antigen and 100 µl/well of poly-L-glutamate (50 µg/ml in TBS) was coated for 2 hours at room temperature. In case of RNP antigen the wells were coated with 100 ul of stock (40 µg/ml in carbonate - bicarbonate buffer) and incubated for desired time period. The plates were washed thrice with TBS-T and the unoccupied sites were blocked with 150 µl of BSA (1.5% in TBS) for six hours at room temperature. The plates were washed once with TBS-T and serially diluted SLE sera were added to wells. After incubation for 2 hours at room temperature and overnight at 4°C. The bound antibodies were assayed by anti-human IgG alkaline phosphatase conjugate using para-nitrophenyl phosphate (PNPP) as colorigenic substrate. The plates were developed for 45-60 min and reaction was stopped by adding 100 ul of 3 M NaOH solution in each well. The absorbance of each well was monitored at 410 nm. Results were expressed as a mean of $A_{test} - A_{control}$.

12. Inhibition ELISA

Antibody specificity was determined by inhibition experiments (Alam et al., 1993, 1995). Briefly varying amount

of inhibitors were mixed with a constant amount of antibody and mixture was incubated for 2 hours at room temperature and overnight at 4°C. The immune complex thus formed was coated on to the wells instead of serum. The remaining steps were same as described earlier.

Results

The most striking feature of human SLE is the presence of circulating antibodies against nDNA (B-conformation). Their level is a direct measure of the disease state. Since immunization with mammalian nDNA have failed to induce anti-DNA antibodies beyond adjuvant level, it has been postulated that DNA modified by physical/chemical/environmental agents is the likely immunogen. Some other structurally altered macromolecule is the stimulus for the production of characteristic autoantibodies to cellular and nuclear components. Among the highly conserved cell components that can be target of the autoimmune reactions are the ribonucleoprotein (RNP) particles. Under oxidative stress these particles might behave as immunogenic trigger for the production of at least a sub-population of SLE autoantibodies while DNA is actually a crossreacting antigen.

Purification and Characterization of Ribonucleoprotein Particles

The goat liver nuclei was chosen as the source of ribonucleoprotein particles in view of its easy availability in bulk besides being a rich source of RNP particles. Isolated nuclei were extracted with STM buffer and nuclear extract was subjected to protein and nucleic acids determinations. Protein, RNA and DNA content of the nuclear extract was found to be 21, 36 and 33.2 percent respectively.

Nuclear extract passed through Sepharose 4B column separated into two peaks (Fig 6). Colorimetric estimation of peak fractions by orcinol reagent revealed 22.8 µg/ml and 13 µg/ml RNA in peak 1 and peak 2 respectively. Fraction of peak 1 and peak 2 were concentrated separately and dialysed against 10 mM sodium phosphate buffer, pH 7.3 containing 50 mM NaCl. The concentrated sample was passed through DEAE Sephacel column and unbound proteins were washed with sodium phosphate buffer. The bound protiens were eluted as single peak (Fig. 7). Fractions were devoid of DNA. After extensive dialysis against 10 mM sodium phosphate buffer, pH 7.3 containing 50 mM sodium chloride the proteins were analyzed on polyacrylamide gel under denaturing conditions. The results shows polypeptide bands in the range of 29 kd to 116 kd (Fig. 8). That the RNA quantitated by orcinol reagent was indeed contributed by RNP particles (and not as contamination) was further confirmed by ethidium bromide induced fluorescence of agarose gel (Fig. 9).

Modification of RNP Particles with Hydroxyl Radical

The ribonucleoprotein particles were irradiated under 254 nm UV light for 30 min at room temperature in the presence of hydrogen peroxide in the molar ratio of 1:100 (RNP:H_2O_2). Excess of hydrogen peroxide from the reaction mixture was removed by extensive dialysis against PBS, pH 7.4. The UV absorption spectra of hydroxyl modified RNP particles alongwith

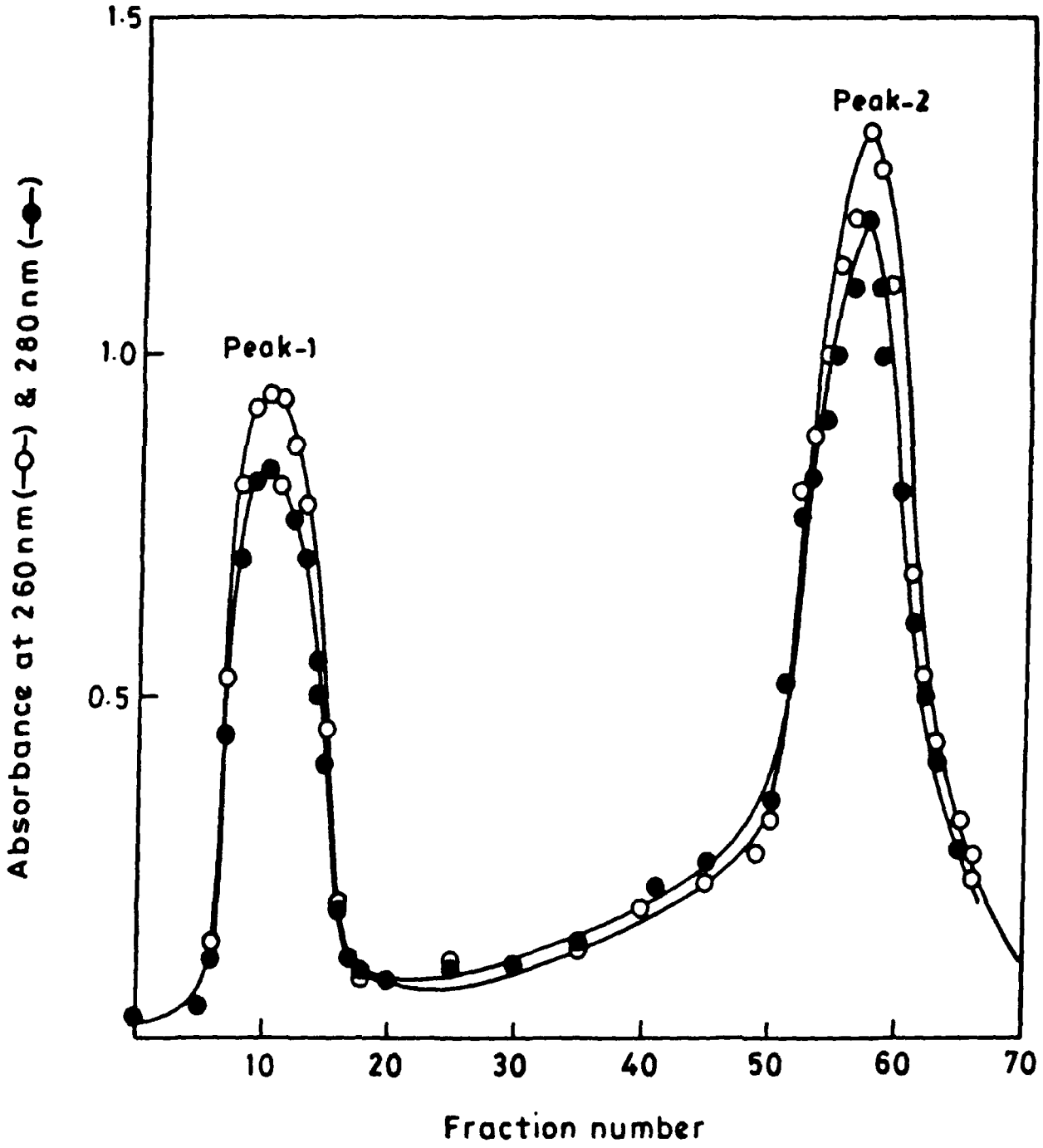


Fig. 6. Elution profile of goat liver nuclear extract passed through Sepharose 4B column. Nuclear extract was loaded on Sepharose 4B column (70 x 2 cm) equilibrated with STM buffer. Three ml fractions were collected and monitored at 260 (—O—) and 280 nm (—●—).

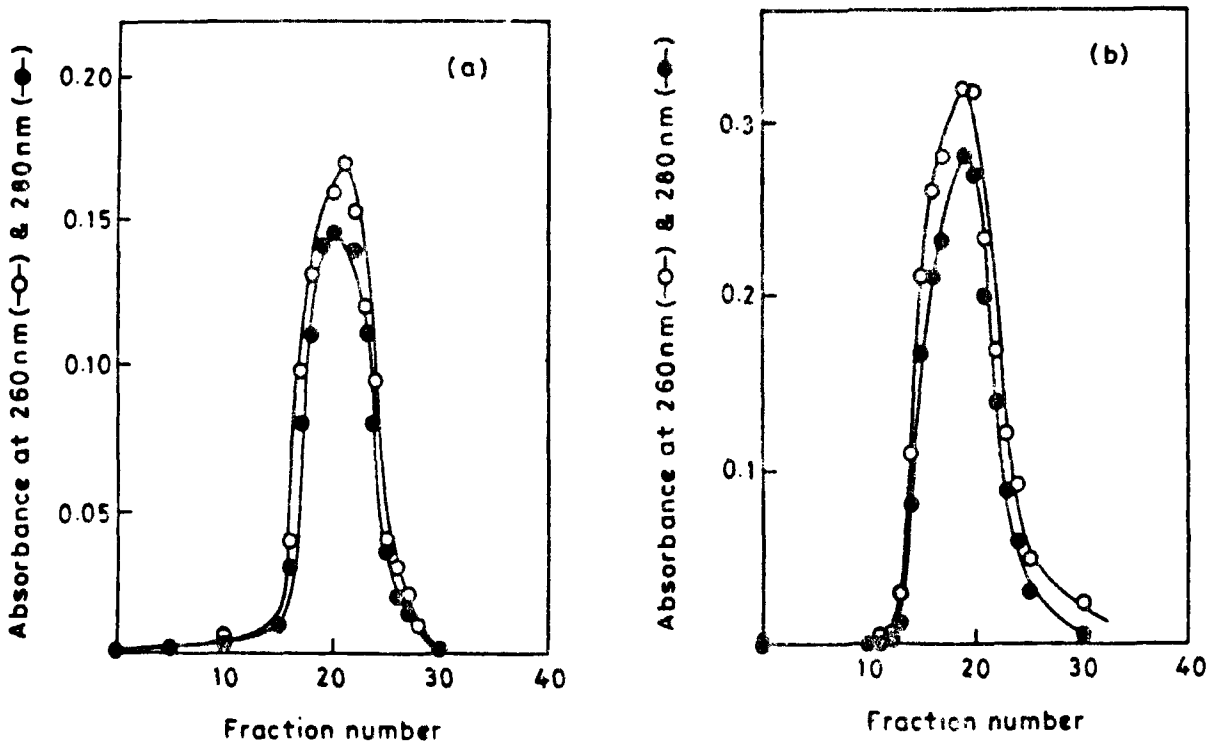


Fig.7(**a**) DEAE-Sephacel ion exchange chromatography of Sepharose 4B fractionated peak 1.

(**b**) DEAE-Sephacel ion exchange chromatography of Sepharose 4B fractionated peak 2.
Concentrated sample of peak 1 and peak 2 fractions were loaded and eluted with 300 mM NaCl.
Three ml fractions were collected and monitored at 260 nm (—O—) and 280 nm (—●—).

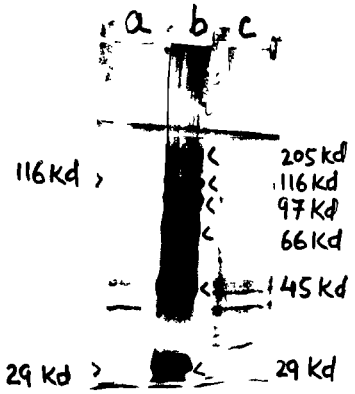


Fig. 8. Banding pattern of native RNP protein antigens in polyacrylamide gel under denaturing conditions. Samples were mixed with 10 mM Tris, pH 8.8 containing 1 percent SDS and 5 percent 2-mercaptoethanol boiled for 3 min and loaded on gradient gel. (a) Peak 2 (b) protein marker (c) Peak 1

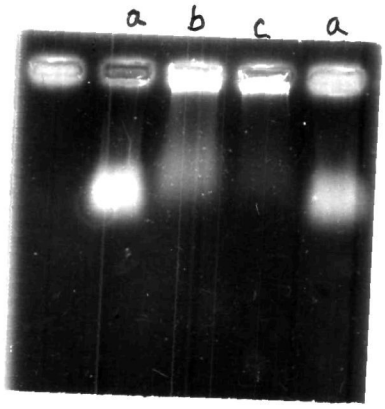


Fig. 9. Submarine agarose gel electrophoresis of RNP particles. (a) Buffalo thymus RNA (b) Peak 1 (c) Peak 2

proper controls was recorded to detect changes in the absorption pattern. UV absorption profile of native RNP particles showed maxima at 270 nm and minima at 255 nm (Fig.10). Irradiation of RNP particles without hydrogen peroxide showed a shoulder around 260 nm while the characteristics λ max at 270 nm of native RNP was lost (Fig.11). On the other hand, RNP-hydrogen peroxide complex not exposed to UV light resulted in almost 35% decrease in absorption at 270 nm. Absorption spectrum of RNP-H_2O_2 complex exposed to UV light showed almost 42% decreased in absorbance at 270 nm. Almost total loss of characteristic maxima was observed (Fig. 10).

Characterization of ROS Modified RNP Particles

Melting characteristics of modified and unmodified RNP particles was monitored between 30°C and 95°C. Increase in absorbance at 280 nm was recorded by heating the samples at a rate of 1.5°C/min. The Tm values of RNP and ROS-RNP particles was found to be 86°C and 94°C respectively (Fig. 12).

To detect the effect of hydroxyl radical on particle proteins, SDS-PAGE of ROS-RNP particles alongwith native RNP particles as control was carried out. The native RNP particles used as control showed polypeptide of molecular weights of 29Kd 32Kd, 35Kd, 40Kd, 45Kd, 55Kd, 65Kd, 99Kd and 116Kd. The banding pattern of ROS-RNP fractionated proteins, as compared to native RNP proteins revealed only five polypeptides of 29Kd, 32kd, 40kd,

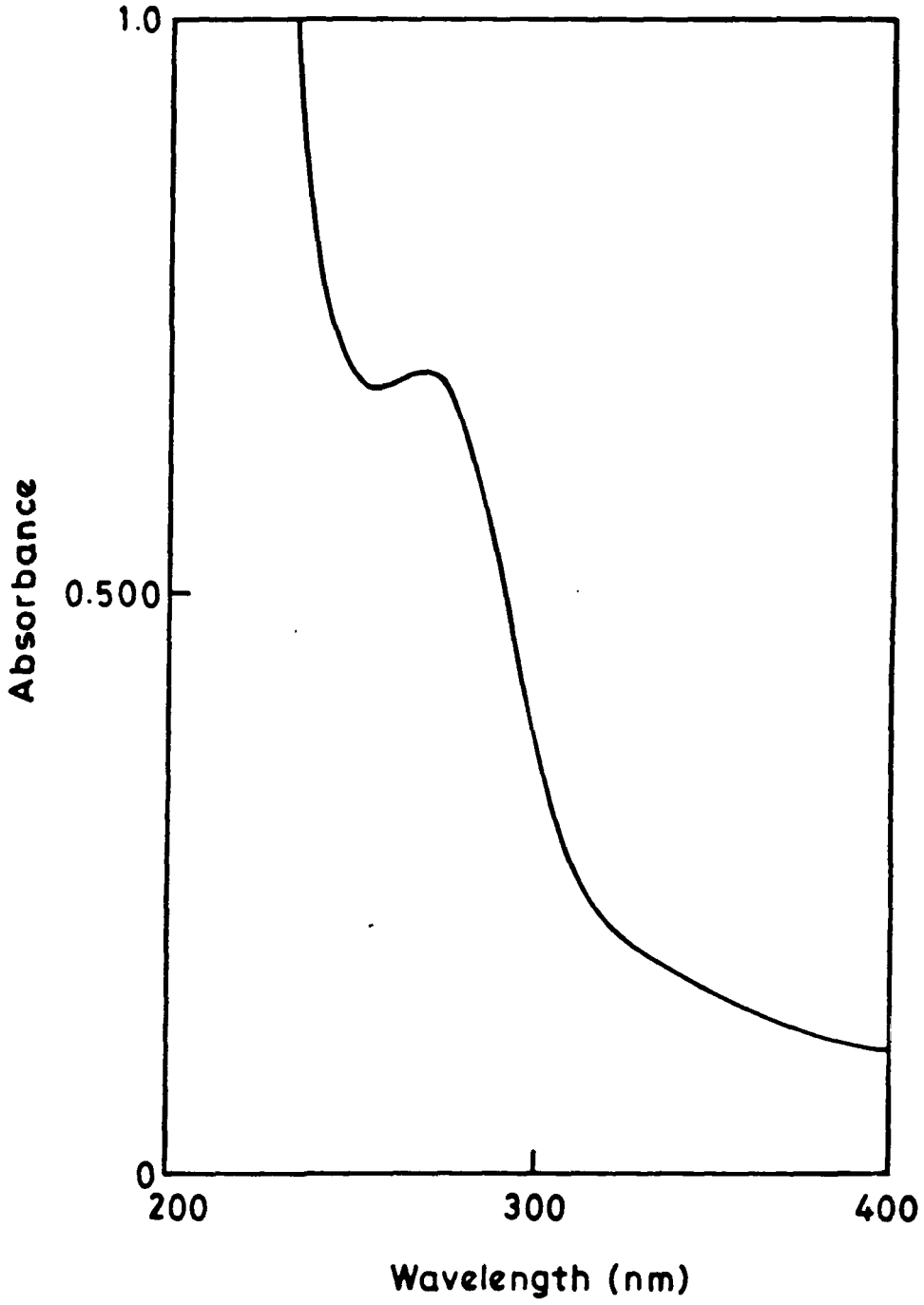


Fig.10. UV absorption profile of native RNP particles.

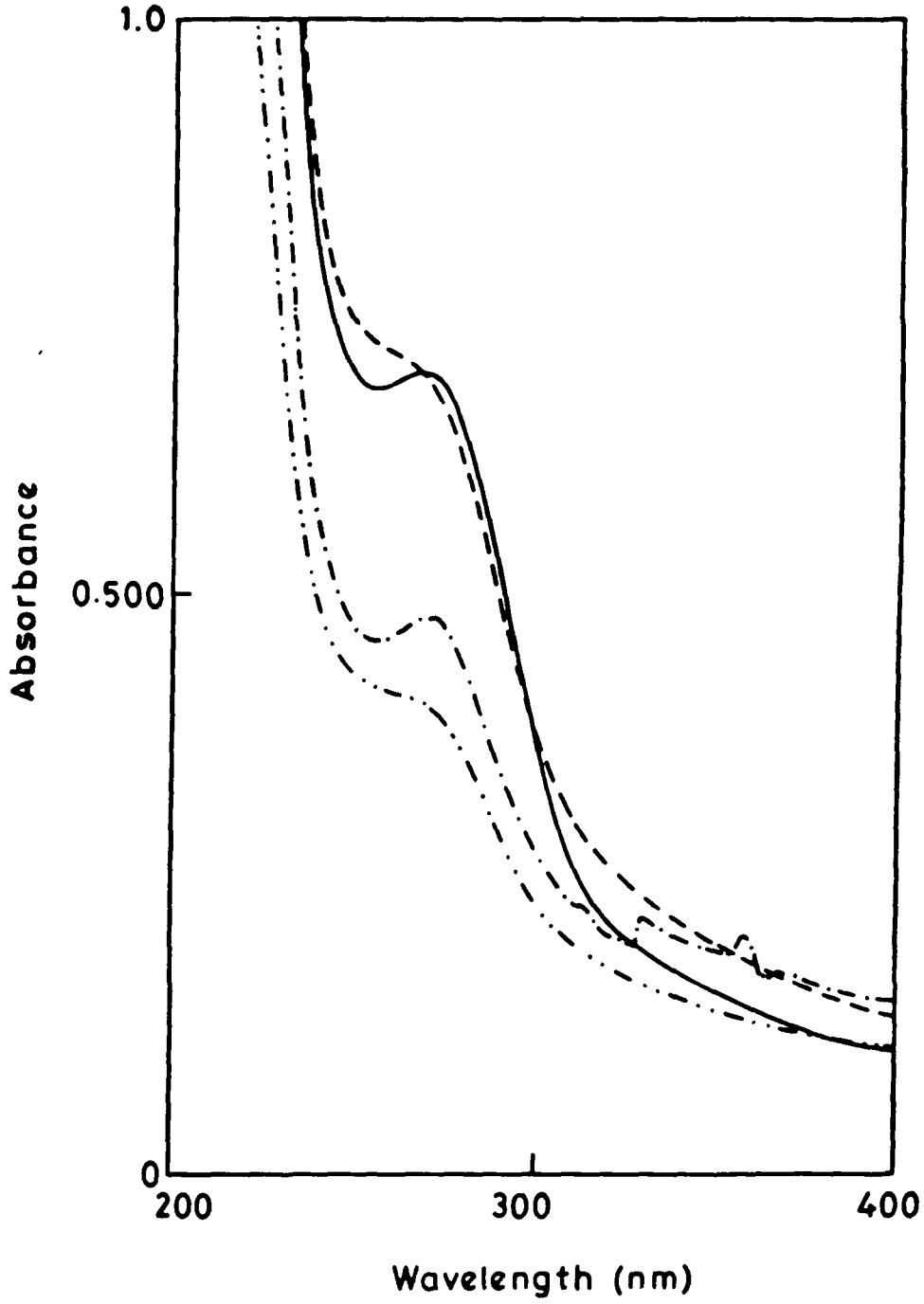


Fig.11. UV absorption characteristics of native RNP and ROS-RNP particles.
(———) native RNP
(– – – –) native RNP+UV irradiation.
(—·—·—) native RNP+Hydrogen peroxide.
(—··—··—) native RNP+Hydrogen peroxide + UV irradiation.

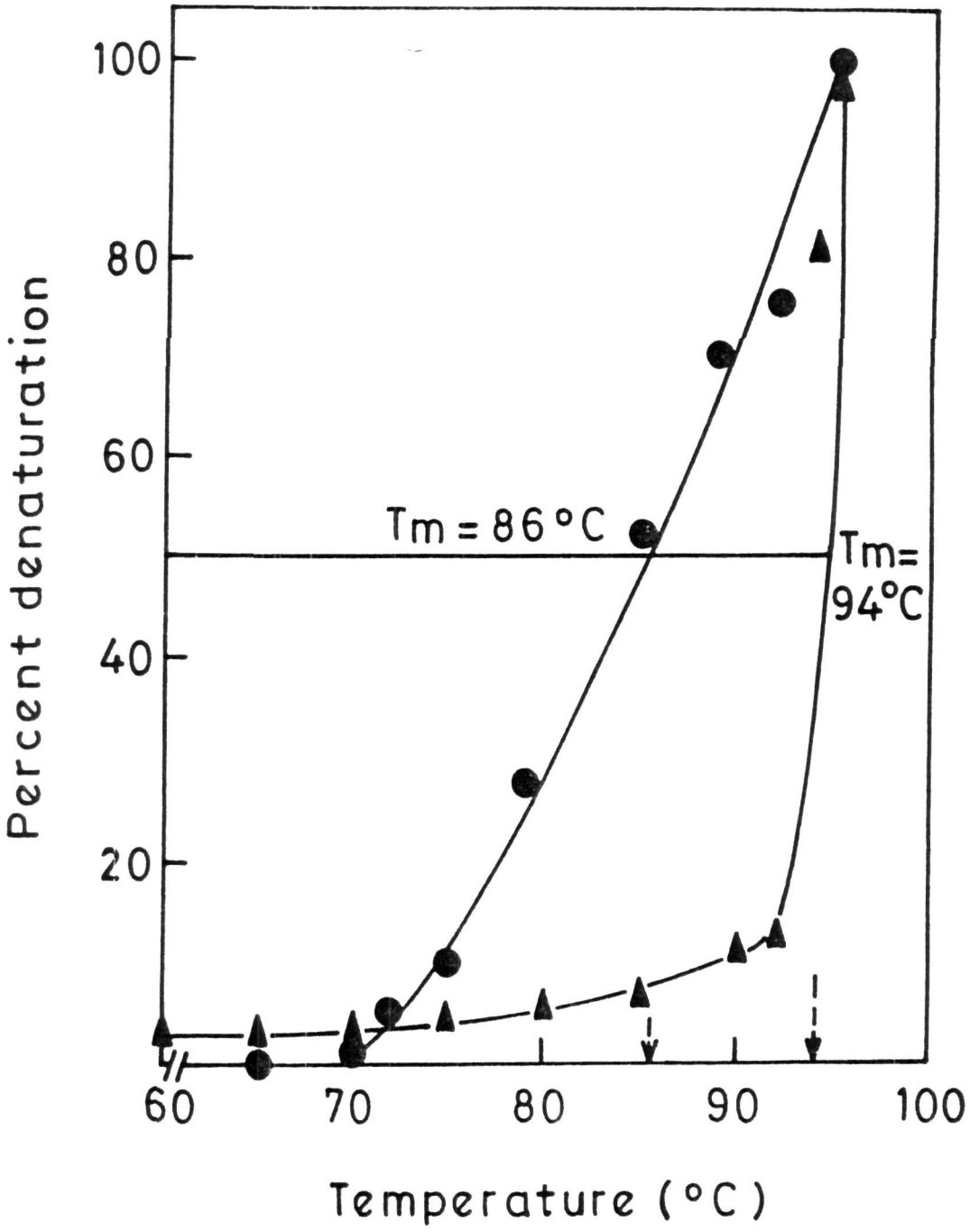


Fig. 12. Melting profile of native and ROS-RNP particles.
(—●—) Native RNP.
(—▲—) ROS-modified RNP

55kd and 65kd respectively (Fig. 13). The disappearance of high molecular weight polypeptides may be due to their fragmentation into smaller peptides which is beyond the detection limit (sensitivity) of Coomassie Brilliant Blue R 250.

Enzyme Immunoassay

The binding studies of RNP and ROS-RNP particles with anti-DNA antibodies positive SLE sera were carried out by direct binding and competition immunoassays. Of the 20 suspected SLE samples, only six sera were found to have moderate to high antibody level and were chosen for further studies. The direct binding and competition ELISA results of above samples are shown in Fig. 14 and Table 1 respectively. Binding of SLE sera was carried out with RNP and ROS-RNP particles coated separately in equal amounts on microtiter plates. Binding of SLE autoantibodies, at 1:100 dilution, with ROS-RNP particles was observed to be higher and of varying degree in Fig. 15. The binding specificity of SLE auto-antibodies to RNP and ROS-RNP particle was further evaluated by competitive enzyme immunoassay on plates coated with native RNP particles. The SLE sera showed inhibition of autoantibody binding with RNP and ROS-RNP particles used as competitor (Fig. 16). The inhibition data reveals that ROS modified RNP particles are not a better competitor for SLE autoantibody as the modification has resulted in decreased inhibition compared to native RNP particles.

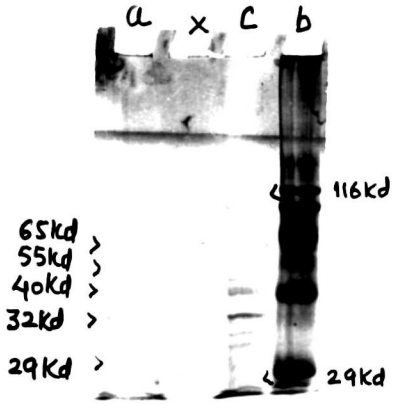


Fig. 13. Polyacrylamide gel electrophoresis of ROS-RNP particles under denaturing conditions.
Samples were mixed with 10 mM Tris, pH 8.8 containing 1 percent SDS and 5 percent 2-mercaptoethanol boiled for 3 min and loaded on gradient gel. (a) ROS-RNP (b) protein marker (c) native RNP.

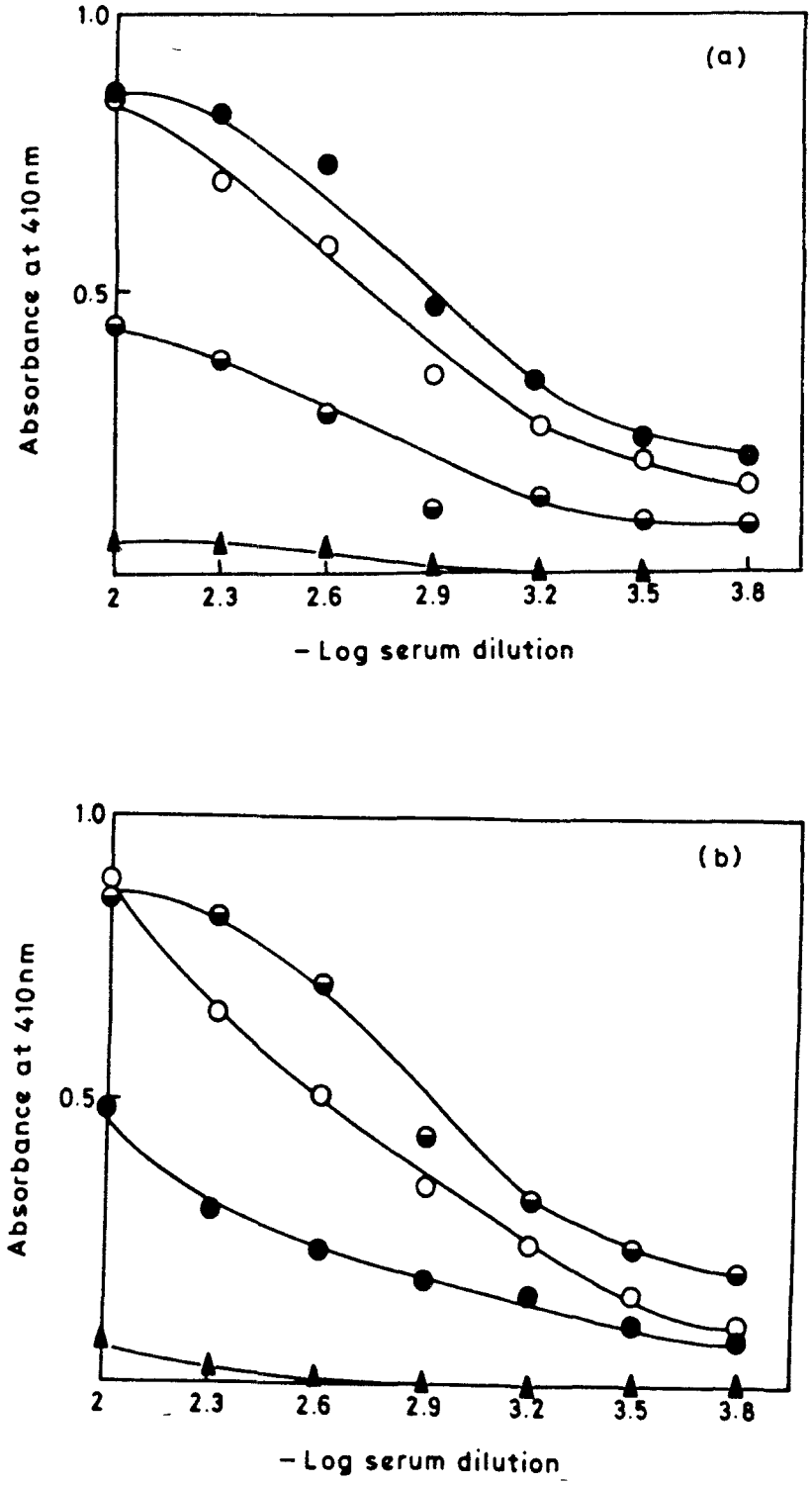


Fig. 14. Direct Binding ELISA of SLE sera. The plate was coated with native DNA.

(a) (—○—) SLE Serum 1, (—●—) SLE Serum 2
(—◐—) SLE Serum 3, (—▲—) Negative control
(b) (—◐—) SLE Serum 4, (—○—) SLE Serum 5
(—●—) SLE Serum 6, (—▲—) Negative control

TABLE.1

Competition ELISA of SLE sera showing the inhibition of DNA-anti-DNA antibodies interaction by native DNA.

SERA NUMBER	IC_{50} (ug/ml)	Minimum percent inhibition
1.	1.6	58.3
2.	3.0	70.74
3.	4.5	75.4
4.	20.0	55.4
5.	22.0	55.6
6.	50.0	51.07

IC_{50} = Competitor concentration for 50 percent inhibition.

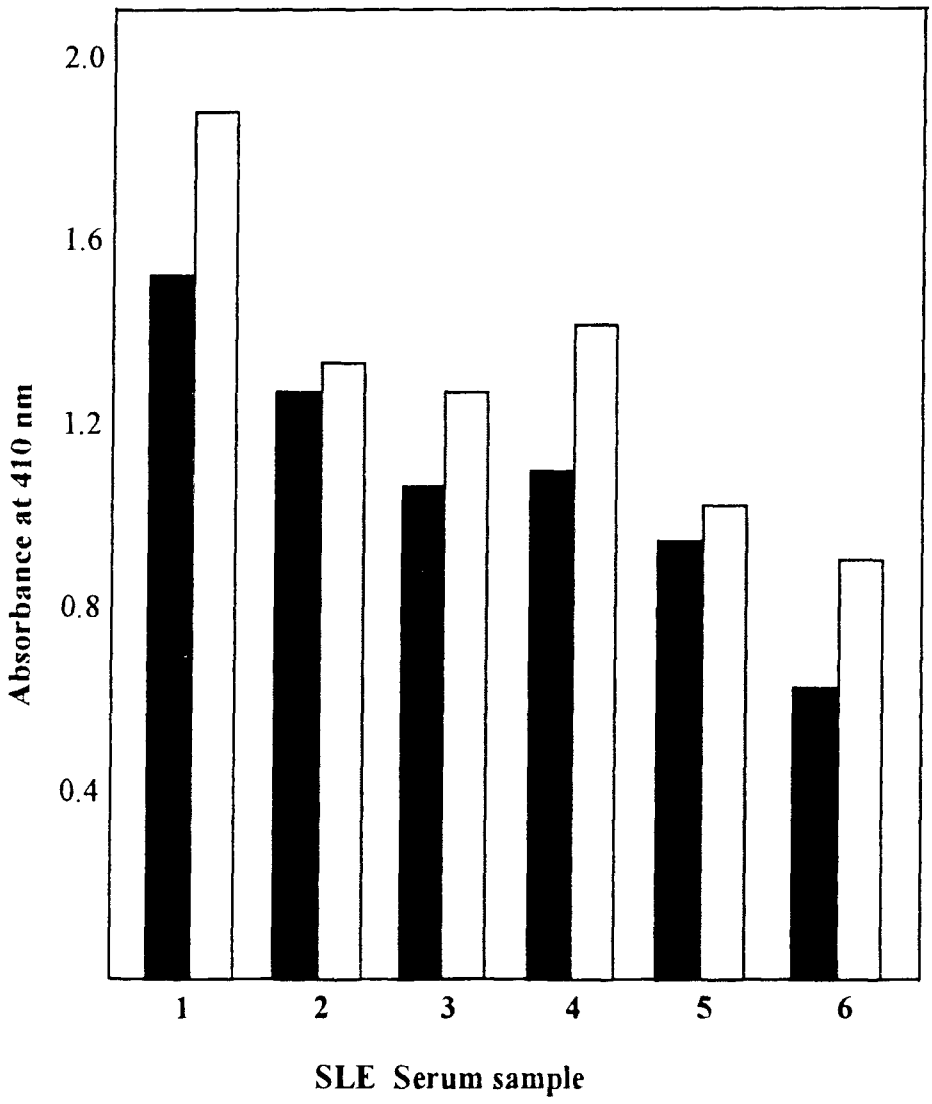


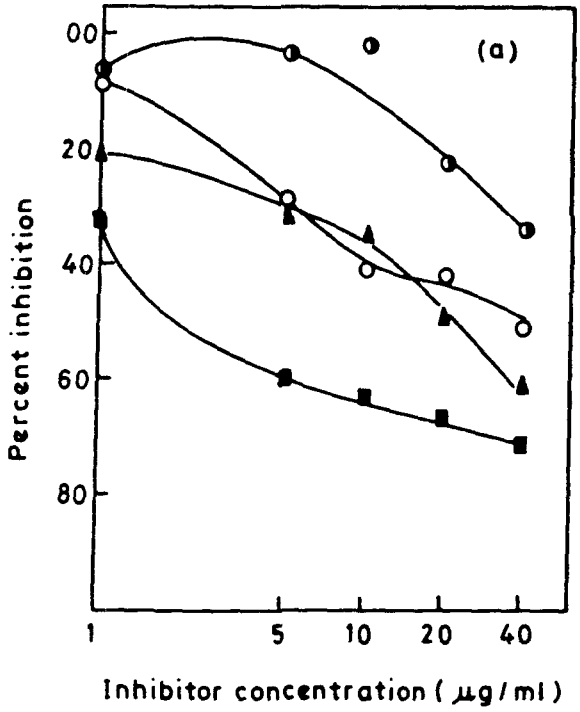
Fig. 15. Direct binding ELISA of SLE sera with native RNP (■) and ROS-RNP (□).

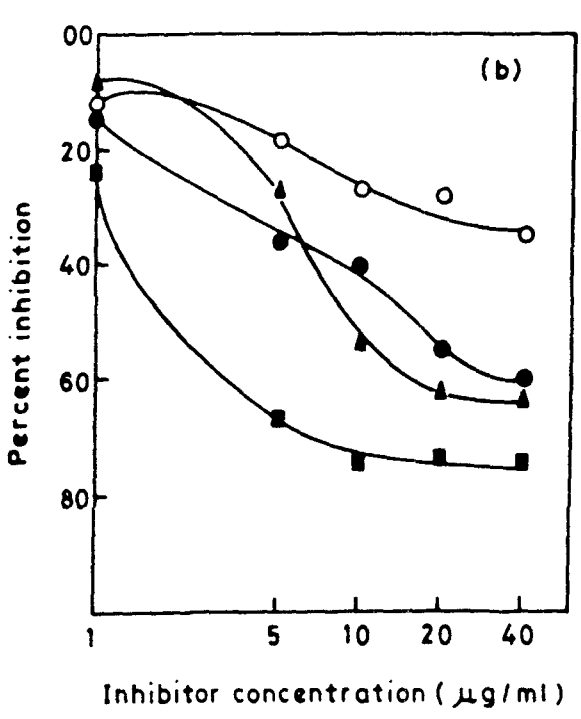
Fig. 16. Competition ELISA showing the inhibition of various SLE autoantibody activity with native and ROS-RNP particles.

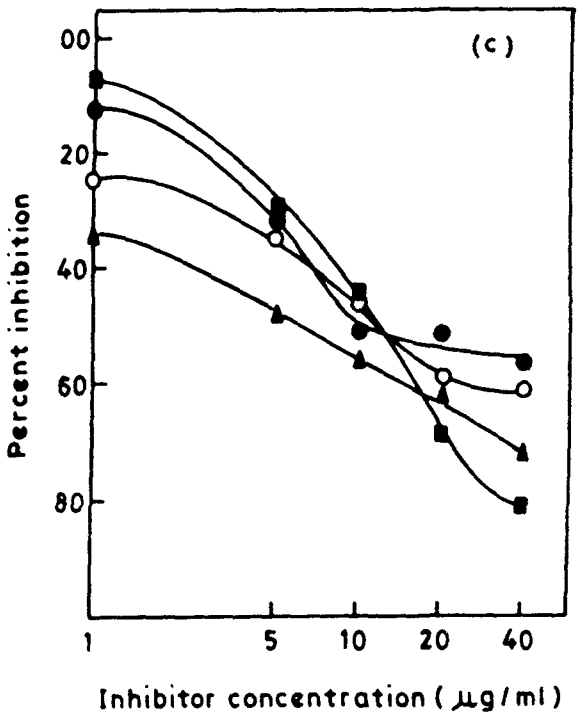
(**a**) Serum 1: (—○—) native RNP particles, (—◑—) ROS-RNP Particles.
Serum 2: (—■—) native RNP particles, (—▲—) ROS-RNP Particles.

(**b**) Serum 3: (—●—) native RNP particles, (—○—) ROS-RNP Particles.
Serum 4: (—■—) native RNP particles, (—▲—) ROS-RNP Particles.

(**c**) Serum 5: (—○—) native RNP particles, (—●—) ROS-RNP Particles.
Serum 6: (—■—) native RNP particles, (—▲—) ROS-RNP Particles.


00
20
40
60
80
(a)
Percent inhibition
1
5
10
20
40
Inhibitor concentration (µg/ml)


00
20
40
60
80
(b)
Percent inhibition
1
5
10
20
40
Inhibitor concentration (µg/ml)


00
20
40
60
80
(c)
Percent inhibition
1
5
10
20
40
Inhibitor concentration (µg/ml)

Discussion

Systemic lupus erythematosus (SLE) is an autoimmune disease of unknown etiology characterized by the presence of antibodies to numerous self components which include nuclear and cytoplasmic antigens (Tan, 1989; Pisetsky, 1994). Native DNA (B-conformation) is a poor immunogen and antibodies are rarely induced upon immunization (Madaio et al., 1984). However, a number of synthetic polynucleotides and conformational variants of DNA including modified structures have been shown to be potent immunogen (Hasan et al., 1991; Alam and Ali, 1992; Alam et al., 1995). Earlier studies have suggested that circulating antibodies against a variety of cellular components found in patients with SLE and mixed connective tissue diseases (MCTD) may serve as useful tool for studying structure and function of small ribonucleoprotein (snRNP) complex (Lerner and Steitz, 1981). Autoantibodies binding to ribonucleoprotein (RNP) particles are often found in patient with SLE and SLE overlap syndrome (Tan, 1989).

In recent years there has been considerable interest on reactive oxygen species (ROS) and the damage caused to living system in their presence (Halliwell, 1987; Yu, 1994). Role of ROS in the development of autoimmune diseases, cancer, diabetes and other human diseases has been the subject of recent studies

(Ames, 1983; Meneghini, 1988; Lunec et al., 1994). Among the reactive oxygen intermediates, hydroxyl radical is considered to be extremely dangerous species reacting with many biological macromolecules in its vicinity. Effect of hydroxyl radical on the antigenic structure of nuclear and cytoplasmic protein has been thoroughly discussed to suggest their possible role in SLE, cancer and other degenerative diseases (Stadtman, 1990; Stadtman and Oliver 1991). The free radical damage to proteins consisted mainly crosslinking and fragmentation. In general, aromatic amino acids and cysteine residues are more susceptible to free radical attack (Grossweiner and Smith, 1989).

Nuclear extract passed through Sepharose 4B column followed by DEAE Sephacel chromatography revealed polypeptide bands of 29Kd, 32Kd, 35Kd, 40Kd, 45Kd, 55Kd, 65Kd, 99Kd and 116Kd in polyacrylamide gel under denaturing conditions. There has been considerable variation in the published reports on the molecular weight of RNP particles. The demonstration of number of polypeptide bands in our case is in close agreement with earlier reports on RNP particles isolated from other sources (Lerner and Steitz, 1979; White et al., 1981; Billings et al., 1982). Studies from this laboratory have reported the polypeptides of molecular weight 14Kd, 30Kd, 70Kd and 80Kd (Ishaq and Ali, 1983).

The UV absorption profile of native RNP particles showed maxima at 270 nm and minima at 255 nm. The RNP-hydrogen peroxide

complex, not exposed to UV light, resulted in almost 35% decrease in absorbance at 270 nm. Absorption spectra of RNP-hydrogen peroxide exposed to UV light of 254 nm showed almost 42% decrease in absorbance. The appearance of shoulder at the wavelength maxima coupled with decreased absorbance appears to be a consequence of hydroxyl radical modifying effect on RNP particles. Furthermore, since aromatic amino acids of proteins are the major chromophores responsible for UV light absorption, the 42% decrease in absorbance value of ROS-RNP could possibly be attributed to the modification of RNP particles. Moreover, the disappearance of some high molecular weight polypeptides of ROS-RNP particles, compared to native RNP particles, appears to be due to fragmentation of the polypeptides by reactive oxygen species. And this fragmentation has produced peptides of such small molecular weight that they can not be detected on our conventional polyacrylamide gel usig Coomassie Brilliant Blue R 250 as staining dye.

Thermal denaturation studies of ROS-RNP particles indicated melting temperature (Tm) of 94°C. The Tm of native RNP particles was found to be 86°C. The increased melting temperature of ROS-RNP particles may be the result of crosslinking of RNA with particle proteins. Studies have shown that RNP particles exposed to 254 nm UV irradiation in the presence of hydrogen peroxide leads to direct crosslinking of ribonucleoprotein to snRNAs (Woppmann et al., 1988).

Anti-DNA positive SLE sera were selected on the basis of specificity with native DNA in competition ELISA. Binding of anti-DNA positive sera with native RNP and ROS-RNP particles was studied employing direct binding and competitive immunoassay. In direct biding assay on ELISA plates coated separately with equal amount of native RNP and ROS-RNP the SLE autoantibodies were found to show more binding with ROS-RNP particles with each samples. Whether this binding was specific is debatable as there are many non-specific proteins in serum which could show binding. To sustain the results of direct binding assay, competition ELISA was performed on ELISA plates coated with RNP particles. RNP and ROS-RNP were used as competitor for SLE anti-DNA autoantibodies. Result analysis revealed that modification of RNP particles by hydroxyl radical has destroyed some of the antigenic property of the particles as the extent of inhibition with ROS-RNP particles was low compared to native RNP particles. The decreased inhibition with ROS-RNP particles could be explained as follows :

1. As a result of crosslinking, some epitopes of RNP particles are being masked and therefore not available for antibody binding.
2. There appears to be loss of some particle epitopes as a result of hydroxyl attack on aromatic amino acids.
3. The ROS-modified RNP particles appears not involved in disease initiation or progression.

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